BACTERIAL REVERSE MUTATION TEST

Report

CONFIDENTIAL

[redacted]

[redacted]

BACTERIAL REVERSE MUTATION TEST

[redacted]

CONTENTS

CONTENTS (continued)

Page

TABLES

APPENDICES

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards, with the exception stated below, and I consider the data generated to be valid.

The UK Good Laboratory Practice Regulations (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994).

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM (98) 17.

EC Commission Directive 2004/10/EC of 11 February 2004 (Official Journal No. L 50/44).

These principles of Good Laboratory Practice are accepted by the regulatory authorities of the United States of America and Japan on the basis of intergovernmental agreements.

In line with normal practice in this type of short-term study, the protocol did not require analysis of the dose form.

QUALITY ASSURANCE STATEMENT

The following inspections and audits have been carried out in relation to this study:

Study Phase	Date(s) of Inspection	Date of Reporting to Study Director and Management
Protocol Audit	28 April 2004	28 April 2004
Report Audit	11 – 18 June 2004	18 June 2004

Process based inspections: At or about the time this study was in progress inspections of procedures employed on this type of study were carried out. These were conducted and reported to appropriate Company Management as indicated below:

Process Based Inspections	Date(s) of Inspection	Date of Reporting to Management
Formulation and Treatment	3 June 2004	4 June 2004
Plate Scoring	17 May 2004	17 May 2004

In addition, an inspection of the facility where this study was conducted was carried out on an annual basis. These inspections were promptly reported to Company Management.

.....

Date

CONTRIBUTING SCIENTISTS

SUMMARY

In this *in vitro* assessment of the mutagenic potential of [redacted] histidine-dependent auxotrophic mutants of *Salmonella typhimurium*, strains TA1535, TA1537, TA98 and TA100, and a tryptophan-dependent mutant of *Escherichia coli*, strain WP2 *uvr*A (pKM101), were exposed to [redacted] diluted in dimethyl sulphoxide (DMSO). DMSO was also used as a negative control.

Two independent mutation tests were performed in the presence and absence of liver preparations from Aroclor 1254-treated rats (S9 mix). The first test was a standard plate incorporation assay; the second included a pre-incubation stage.

Concentrations of [redacted] up to 5000 µg/plate were tested. This is the standard limit concentration recommended in the regulatory guidelines that this assay follows. Other concentrations used were a series of *ca* half-$\log_{10}$ dilutions of the highest concentration. No signs of toxicity were observed towards the tester strains in either mutation test.

No evidence of mutagenic activity was seen at any concentration of [redacted] in either mutation test.

The concurrent positive controls demonstrated the sensitivity of the assay and the metabolising activity of the liver preparations.

It is concluded that [redacted] showed no evidence of mutagenic activity in this bacterial system under the test conditions employed.

INTRODUCTION

This report describes a study designed to assess [redacted] for its ability to cause point (gene) mutation in *Salmonella typhimurium* and *Escherichia coli*. The study was conducted in compliance with the following guidelines:

OECD Guidelines for the Testing of Chemicals. (1997) Genetic Toxicology: Bacterial Reverse Mutation Test, Guideline 471.

EC Commission Directive 2000/32/EC Annex 4D-B.13/14. Mutagenicity - Reverse mutation test in bacteria. No. L 136/57.

US EPA (1998) Health Effects Test Guidelines. OPPTS 870.5100 Bacterial reverse mutation test. EPA 712-C-98-247.

Japanese Ministry of Agriculture, Forestry and Fisheries. Test Data for Registration of Agricultural Chemicals, 12 Nohsan No. 8147, Agricultural Production Bureau, November 24, 2000.

Joint Directives of JEPA, JMHW and JMITI. (31 October 1997) Kanpoan No. 287, Eisei No. 127 and Kikyoku No. 2 (31 October 1997).

JMHW Genotoxicity Testing Guideline, PAB Notification No. 1604 (1 November 1999).

Official Notice of J MOL (8 February 1999).

ICH (1996) Guideline S2A: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. PAB/PCD Notification No. 444.

ICH (1998) Guideline S2B: Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. PMSB/ELD Notification No. 554.

The *in vitro* technique described by Ames and his co-workers (Ames, McCann and Yamasaki 1975, Maron and Ames 1983) enables the mutagenic effect of a test substance to be determined by exposing specially selected strains of *Salmonella typhimurium* to the test substance and so forms part of the general requirements for registration.

Normally *S. typhimurium* is capable of synthesising the essential amino acid, histidine, but the mutant strains used in this test are incapable of this function. When these strains are exposed to a mutagen, reverse mutation to the original histidine-independent form takes place in a proportion of the population. These are referred to as revertants, and are readily detected by their ability to grow and form colonies on a histidine-deficient medium (supplemented with biotin, since these strains are also incapable of biotin synthesis).

A technique based on similar principles has also been described by Green (1984). This system employs mutant strains of *Escherichia coli* that are incapable of synthesising the amino acid, tryptophan, which is required for growth.

The strains used carry additional mutations that render them more sensitive to mutagens. The *S. typhimurium* strains have a defective cell coat, which allows greater permeability of test substances into the cell. All the strains are deficient in normal DNA repair processes. In addition, three of them possess a plasmid (pKM101) which introduces an error-prone repair process, resulting in increased sensitivity to some mutagens.

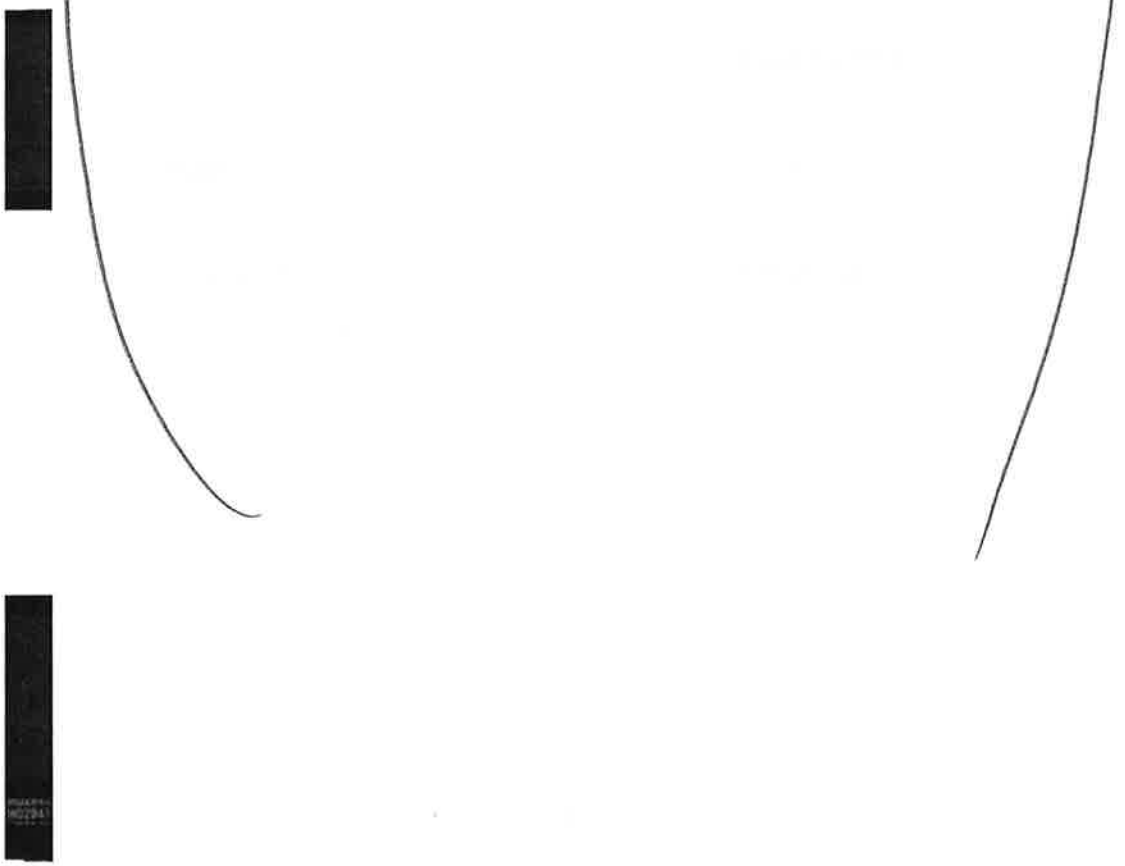
Some substances do not exert a mutagenic effect until they have been metabolised by enzyme systems not available in the bacterial cell. Therefore, the bacteria and test substance are incubated in both the absence and presence of a supplemented liver homogenate fraction (S9 mix).

The protocol was approved by [redacted] on 7 April 2004, by the Sponsor on 16 April 2004 and by the Study Director on 26 April 2004.

[redacted]

[redacted]

TEST SUBSTANCE



EXPERIMENTAL PROCEDURE

BACTERIAL STRAINS

The genotype of each bacterial strain used in this study is outlined in the following table:

Species	Strain	Genotype	
Salmonella typhimurium	TA1535	*his*G46	*rfa* Δ *uvr*B
Salmonella typhimurium	TA1537	*his*C3076	*rfa* Δ *uvr*B
Salmonella typhimurium	TA98	*his*D3052	*rfa* Δ *uvr*B (pKM101)
Salmonella typhimurium	TA100	*his*G46	*rfa* Δ *uvr*B (pKM101)
Escherichia coli	WP2 *uvr*A (pKM101)	*trp*E	Ochre *uvr*A (pKM101)

Note: The deletion (Δ) through *uvr*B also affects the nitrate reductase (*chl*A) and biotin (*bio*) genes

These strains were used to detect base changes and frameshift mutations as follows:

base change mutagens: *S. typhimurium* TA1535 and TA100, and *E. coli* WP2 *uvr*A (pKM101).

frameshift mutagens: *S. typhimurium* TA1537, TA98 and TA100.

The strains of *S. typhimurium* were obtained from the National Collection of Type Cultures, London, England.

The strain of *E. coli* was obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland.

Batches of the strains were obtained from master stocks held in liquid nitrogen. The test batches were stored as aliquots of nutrient broth cultures at -80°C. [redacted] was added to the cultures at 8% v/v as a cryopreservative. Each batch of frozen strain was tested, where applicable, for cell membrane permeability (*rfa* mutation), sensitivity to UV light, and the pKM101 plasmid, which confers resistance to ampicillin. The responses of the strains to a series of diagnostic mutagens were also assessed.

For use in tests, an aliquot of frozen culture was added to 25 mL of nutrient broth and incubated, with shaking, at 37°C for 10 hours. These cultures were intended to provide approximately 10^9 cells per mL, which were measured by spreading aliquots (0.1 mL) of a 10^{-6} dilution of the overnight cultures on the surface of plates of nutrient agar and counting the resultant colonies.

POSITIVE CONTROLS

In the absence of S9 mix

Identity:	Sodium azide
CAS No.:	26628-22-8
Supplier:	Sigma Chemical Company
Lot number:	77H0079
Purity:	min. 99.5%
Appearance:	White powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	0.5 µg/plate for strains TA1535 and TA100

Identity:	9-Aminoacridine
CAS No.:	90-45-9
Supplier:	Sigma Chemical Company
Lot number:	106F-06682
Purity:	> 97%
Appearance:	Yellow powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	50 µg/plate for strain TA1537

Identity:	2-Nitrofluorene
CAS No.:	607-57-8
Supplier:	Aldrich Chemical Company
Lot number:	508447-022
Purity:	98%
Appearance:	Beige powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	1 µg/plate for strain TA98

Identity:	2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)
CAS No.:	3688-53-7
Supplier:	Wako Pure Chemical Industries Ltd.
Lot number:	WAP 0369
Purity:	98-102%
Appearance:	Red powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	0.05 µg/plate for strain WP2 *uvr*A (pKM101)

In the presence of S9 mix

Identity:	2-Aminoanthracene
CAS No.:	613-13-8
Supplier:	Aldrich Chemical Company
Lot number:	11997-098
Purity:	96%
Appearance:	Green powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	2 µg/plate for strain TA1535 10 µg/plate for strain WP2 *uvr*A (pKM101)

Identity:	Benzo[a]pyrene
CAS No.:	50-32-8
Supplier:	Aldrich Chemical Company
Lot number:	17776-111
Purity:	98%
Appearance:	Yellow powder
Solvent:	DMSO (Aldrich, A.C.S. spectrophotometric grade)
Concentration:	5 µg/plate for strains TA1537, TA98 and TA100

PREPARATION OF S9 FRACTION

Species:	Rat
Sex:	Male
Strain:	Sprague-Dawley derived
Source:	Charles River UK Ltd.
Age:	7-8 weeks
Weight:	<300 g

S9 fraction was prepared according to the method described by Ames, McCann and Yamasaki (1975). Mixed function oxidase systems in the rat livers were stimulated by Aroclor 1254, administered as a single intraperitoneal injection in corn oil at a dosage of 500 mg/kg body weight. On the fifth day after injection, following overnight fasting, the rats were killed by cervical dislocation and their livers aseptically removed.

The following steps were carried out on ice under aseptic conditions. The livers were placed in 0.15 M KCl (3 mL KCl : 1 g liver) before being transferred to a Potter-Elvehjem homogeniser. Following preparation, the homogenate was centrifuged at 9000 *g* for 10 minutes at 0-4°C. The supernatant fraction (S9 fraction) was dispensed into aliquots and stored at -80°C or below. Each batch of S9 fraction was tested for sterility and efficacy.

Date of preparation: 4 February & 2 March 2004

PREPARATION OF S9 MIX

The S9 mix contained: S9 fraction (10% v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADPH (4 mM) and NADH (4 mM) in water. All the cofactors were filter-sterilised before use.

SELECTION OF VEHICLE AND FORMULATION OF TEST SUBSTANCE

The solubility of s assessed at 50 mg/mL in . in which it dissolved. Aldrich, ACS spectrophotometric grade, lot no. JA 06941 EA) was, therefore, used as the vehicle for this study.

All concentrations cited in this report are expressed in terms of the Synocure 892 BA 70 sample as received.

MUTATION TEST PROCEDURE

First test

70 was added to cultures of the five tester strains at seven concentrations separated by *ca* half-log_{10} intervals. The highest concentration of ited was 50 mg/mL in the chosen vehicle, which provided a final concentration of 5000 µg/plate. This is the standard limit concentration recommended in the regulatory guidelines that this assay follows. The negative control was the chosen vehicle, The appropriate positive controls were also included.

Aliquots of 0.1 mL of the test substance solutions, positive control or negative control were placed in glass vessels. S9 mix (0.5 mL) or 0.1 M pH 7.4 phosphate buffer (0.5 mL) was added, followed by 0.1 mL of a 10 hour bacterial culture and 2 mL of agar containing histidine (0.5 mM), biotin (0.5 mM) and tryptophan (0.5 mM). The mixture was thoroughly shaken and overlaid onto previously prepared Petri dishes containing 25 mL minimal agar. Each Petri dish was individually labelled with a unique code corresponding to a sheet, identifying the contents of the dish. Three Petri dishes were used for each concentration. Plates were also prepared without the addition of bacteria in order to assess the sterility of the test substance, S9 mix and sodium phosphate buffer. All plates were incubated at 37°C for *ca* 72 hours. After this period, the appearance of the background bacterial lawn was examined and revertant colonies counted using a Domino automated colony counter.

Any toxic effects of the test substance would be detected by a substantial reduction in revertant colony counts or by the absence of a complete background bacterial lawn. In the absence of any toxic effects, the maximum concentration selected for use in the second test would be the same as that used in the first. If toxic effects were observed, a lower concentration might be chosen, ensuring that signs of bacterial inhibition were present at this maximum concentration. Ideally, a minimum of four non-toxic concentrations should be obtained. If precipitate were observed on the plates at the end of the incubation period, at least four non-precipitating dose levels should be obtained, unless otherwise justified by the Study Director.

Second test

As a clear negative response was obtained in the first test, a variation to the test procedure was used for the second test. The variation used was the pre-incubation assay in which the tubes, which contained mixtures of bacteria, buffer or S9 mix and test dilution, were incubated at 37°C for 30 minutes with shaking before the addition of the agar overlay. The maximum concentration chosen was again 5000 µg/plate, but only five concentrations were used.

STABILITY AND FORMULATION ANALYSIS

The stability of the stability and homogeneity of in the vehicle were not determined as part of this study. Analysis of achieved concentration was not performed as part of this study.

ASSESSMENT OF RESULTS

Acceptance criteria

For a test to be considered valid, the mean of the vehicle control revertant colony numbers for each strain should lie within or close to the 99% confidence limits of the current historical control range of the laboratory unless otherwise justified by the Study Director. The historical range is maintained as a rolling record over a maximum of five years. Also, the positive control compounds must induce an increase in mean revertant colony numbers of at least twice (three times in the case of strains TA1535 and TA1537) the concurrent vehicle controls. Viable cell counts in the 10-hour bacterial cultures must be at least 10^9/mL.

Analysis of data

The mean number and standard deviation of revertant colonies are calculated for all groups. The means for all treatment groups are compared with those obtained for the vehicle control groups.

Criteria for assessing mutagenic potential

If exposure to a test substance produces a reproducible increase in revertant colony numbers of at least twice (three times in the case of strains TA1535 and TA1537) the concurrent vehicle controls, with some evidence of a positive dose-response relationship, it will be considered to exhibit mutagenic activity in this test system. No statistical analysis will be performed.

If exposure to a test substance does not produce a reproducible increase in revertant colony numbers, it will be considered to show no evidence of mutagenic activity in this test system. No statistical analysis will be performed.

If the results obtained fail to satisfy the criteria for a clear "positive" or "negative" response, even after additional testing, the test data may be subjected to analysis to determine the statistical significance of any increases in revertant colony numbers. The statistical procedures used will be those described by Mahon *et al* (1989) and will usually be Dunnett's test followed, if appropriate, by trend analysis. Biological importance should always be considered along with statistical significance. In general, treatment-associated increases in revertant colony numbers below two or three times the vehicle controls (as described above) will not be considered biologically important. It should be noted that it is acceptable to conclude an equivocal response if no clear results can be obtained.

Occasionally, these criteria may not be appropriate to the test data and, in such cases, the Study Director will use his/her scientific judgement.

DEVIATIONS FROM PROTOCOL

There were no deviations from the protocol.

MAINTENANCE OF RECORDS

All raw data, samples and specimens (if appropriate) arising from the performance of this study will remain the property of the Sponsor. Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of.

All other samples and specimens and all raw data will be retained by
in its archive for a period of five years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

RESULTS

The results obtained with [redacted] and positive control compounds are presented in Tables 1 to 10. The mean values quoted have been corrected to the nearest whole number.

The absence of colonies on sterility check plates confirmed the absence of microbial contamination.

The total colony counts on nutrient agar plates (see Tables) confirmed the viability and high cell density of the cultures of the individual organisms.

The mean revertant colony counts for the vehicle controls were within the 99% confidence limits of the current historical control range of the laboratory (Appendix 1). Appropriate positive control chemicals (with S9 mix where required) induced substantial increases in revertant colony numbers with all strains, confirming sensitivity of the cultures and activity of the S9 mix.

[redacted]

FIRST TEST

No evidence of toxicity was obtained following exposure to [redacted] maximum exposure concentration of 5000 µg/plate was, therefore, selected for use in the second test.

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to [redacted] at any concentration up to 5000 µg/plate in either the presence or absence of S9 mix.

SECOND TEST

No evidence of toxicity was obtained following exposure to [redacted]

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to [redacted] at any concentration up to 5000 µg/plate in either the presence or absence of S9 mix.

[redacted]

CONCLUSION

It is concluded that [redacted] showed no evidence of mutagenic activity in this bacterial system under the test conditions employed.

REFERENCES

AMES, B.N., McCANN, J. and YAMASAKI, E. (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test. *Mutation Res.* **31**, 347-364.

GREEN, M.H.L. (1984) Mutagen testing using *trp*$^+$ reversion in *Escherichia coli* in KILBEY, B.J., LEGATOR, M., NICHOLS, W. and RAMEL, C. (Eds.). *Handbook of Mutagenicity Test Procedures. Second edition*, pp.161-187. Elsevier Science Publishers BV, Amsterdam.

MAHON, G.A.T., GREEN, M.H.L., MIDDLETON, B., MITCHELL, I. de G., ROBINSON, W.D. and TWEATS, D.J. (1989) Analysis of data from microbial colony assays in: KIRKLAND, D.J. (Ed.). *UKEMS Sub-committee on Guidelines for Mutagenicity Testing. Report. Part III. Statistical Evaluation of Mutagenicity Test Data*, pp.26-65. Cambridge University Press, Cambridge.

MARON, D.M. and AMES, B.N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutation Res.* **113**, 173-215.

TABLE 1: Results obtained with *S. typhimurium* TA98: test 1

Tester strain: *S. typhimurium* TA98
Test substance
Test No.: 1

Addition		S9 mix + present - absent	Revertant colony counts/plate					Ratio treated/ vehicle
			Individual plate counts			Mean	Standard Deviation	
DMSO	(0.1 mL/plate)	-	36	32	39	36	4	-
Test substance	(5 µg/plate)	-	36	31	36	34	3	0.9
Test substance	(15 µg/plate)	-	30	30	0 a	30	0	0.8
Test substance	(50 µg/plate)	-	36	22	42	33	10	0.9
Test substance	(150 µg/plate)	-	31	29	23	28	4	0.8
Test substance	(500 µg/plate)	-	20	24	30	25	5	0.7
Test substance	(1500 µg/plate)	-	23	31	28	27	4	0.8
Test substance	(5000 µg/plate)	-	32	27	32	30	3	0.8
DMSO	(0.1 mL/plate)	+	53	46	48	49	4	-
Test substance	(5 µg/plate)	+	42	55	52	50	7	1.0
Test substance	(15 µg/plate)	+	55	51	43	50	6	1.0
Test substance	(50 µg/plate)	+	42	51	48	47	5	1.0
Test substance	(150 µg/plate)	+	46	49	46	47	2	1.0
Test substance	(500 µg/plate)	+	58	52	50	53	4	1.1
Test substance	(1500 µg/plate)	+	52	44	53	50	5	1.0
Test substance	(5000 µg/plate)	+	64	49	46	53	10	1.1
2-Nitrofluorene	(1 µg/plate)	-	366	328	326	340	23	9.4
Benzo[a]pyrene	(5 µg/plate)	+	821	823	794	813	16	16.6
Viable cell count: 10^{-6} dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	108	128	126	121	11	-
Sterility checks (total colony counts):								
Buffer		-	0	0	0	0	0	-
S9 mix		+	0	0	0	0	0	-
Test substance	(5000 µg/plate)	-	0	0	0	0	0	-

a The bacterial culture was accidentally omitted. Count not included in mean & standard deviation.

TABLE 2: Results obtained with *S. typhimurium* TA98: test 2

Tester strain: *S. typhimurium* TA98
Test substance
Test No.: 2 (with pre-incubation)

Addition		S9 mix + present - absent	Revertant colony counts/plate					Ratio treated/ vehicle
			Individual plate counts			Mean	Standard Deviation	
DMSO	(0.1 mL/plate)	-	37	43	43	41	3	-
Test substance	(50 µg/plate)	-	43	31	36	37	6	0.9
Test substance	(150 µg/plate)	-	38	36	37	37	1	0.9
Test substance	(500 µg/plate)	-	36	34	37	36	2	0.9
Test substance	(1500 µg/plate)	-	34	38	36	36	2	0.9
Test substance	(5000 µg/plate)	-	27	38	36	34	6	0.8
DMSO	(0.1 mL/plate)	+	57	45	45	49	7	-
Test substance	(50 µg/plate)	+	49	41	63	51	11	1.0
Test substance	(150 µg/plate)	+	45	60	43	49	9	1.0
Test substance	(500 µg/plate)	+	45	31	45	40	8	0.8
Test substance	(1500 µg/plate)	+	50	60	46	52	7	1.1
Test substance	(5000 µg/plate)	+	55	45	41	47	7	1.0
2-Nitrofluorene	(1 µg/plate)	-	296	386	285	322	55	7.9
Benzo[a]pyrene	(5 µg/plate)	+	1012	912	909	944	59	19.3
Viable cell count: 10^{-6} dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	123	141	159	141	18	-
Sterility checks (total colony counts):								
Buffer		-	0	0	0	0	0	-
S9 mix		+	0	0	0	0	0	-
Test substance	(5000 µg/plate)	-	0	0	0	0	0	-

TABLE 3: Results obtained with *S. typhimurium* TA100: test 1

Tester strain: *S. typhimurium* TA100
Test substance
Test No.: 1

Addition		S9 mix + present - absent	Revertant colony counts/plate					Ratio treated/ vehicle
			Individual plate counts			Mean	Standard Deviation	
DMSO	(0.1 mL/plate)	-	169	146	154	156	12	-
Test substance	(5 µg/plate)	-	176	141	160	159	18	1.0
Test substance	(15 µg/plate)	-	146	153	136	145	9	0.9
Test substance	(50 µg/plate)	-	125	157	151	144	17	0.9
Test substance	(150 µg/plate)	-	164	125	152	147	20	0.9
Test substance	(500 µg/plate)	-	132	148	129	136	10	0.9
Test substance	(1500 µg/plate)	-	136	151	116	134	18	0.9
Test substance	(5000 µg/plate)	-	126	128	119	124	5	0.8
DMSO	(0.1 mL/plate)	+	154	174	160	163	10	-
Test substance	(5 µg/plate)	+	166	137	158	154	15	0.9
Test substance	(15 µg/plate)	+	126	157	150	144	16	0.9
Test substance	(50 µg/plate)	+	135	154	131	140	12	0.9
Test substance	(150 µg/plate)	+	159	177	143	160	17	1.0
Test substance	(500 µg/plate)	+	162	158	150	157	6	1.0
Test substance	(1500 µg/plate)	+	152	161	125	146	19	0.9
Test substance	(5000 µg/plate)	+	144	157	136	146	11	0.9
Sodium azide	(0.5 µg/plate)	-	818	884	801	834	44	5.3
Benzo[a]pyrene	(5 µg/plate)	+	1018	1011	913	981	59	6.0
Viable cell count: 10^{-6} dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	184	175	176	178	5	-
Sterility checks (total colony counts):								
Buffer		-	0	0	0	0	0	-
S9 mix		+	0	0	0	0	0	-
Test substance	(5000 µg/plate)	-	0	0	0	0	0	-

TABLE 4: Results obtained with *S. typhimurium* TA100: test 2

Tester strain: *S. typhimurium* TA100
Test substance
Test No.: 2 (with pre-incubation)

Addition		S9 mix + present - absent	Revertant colony counts/plate					Ratio treated/ vehicle
			Individual plate counts			Mean	Standard Deviation	
DMSO	(0.1 mL/plate)	-	159	129	143	144	15	-
Test substance	(50 µg/plate)	-	116	107	125	116	9	0.8
Test substance	(150 µg/plate)	-	112	124	131	122	10	0.8
Test substance	(500 µg/plate)	-	108	123	110	114	8	0.8
Test substance	(1500 µg/plate)	-	129	145	126	133	10	0.9
Test substance	(5000 µg/plate)	-	136	122	124	127	8	0.9
DMSO	(0.1 mL/plate)	+	170	162	167	166	4	-
Test substance	(50 µg/plate)	+	159	157	183	166	14	1.0
Test substance	(150 µg/plate)	+	157	168	132	152	18	0.9
Test substance	(500 µg/plate)	+	146	168	137	150	16	0.9
Test substance	(1500 µg/plate)	+	179	161	160	167	11	1.0
Test substance	(5000 µg/plate)	+	159	146	165	157	10	0.9
Sodium azide	(0.5 µg/plate)	-	869	827	796	831	37	5.8
Benzo[a]pyrene	(5 µg/plate)	+	758	685	702	715	38	4.3
Viable cell count: 10^{-6} dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	184	228	215	209	23	-
Sterility checks (total colony counts):								
Buffer		-	0	0	0	0	0	-
S9 mix		+	0	0	0	0	0	-
Test substance	(5000 µg/plate)	-	0	0	0	0	0	-

TABLE 5: Results obtained with *S. typhimurium* TA1535: test 1

Tester strain: *S. typhimurium* TA1535
Test substance:
Test No.: 1

Addition		S9 mix + present - absent	Revertant colony counts/plate					Ratio treated/ vehicle
			Individual plate counts			Mean	Standard Deviation	
DMSO	(0.1 mL/plate)	-	15	19	21	18	3	-
Test substance	(5 µg/plate)	-	20	12	21	18	5	1.0
Test substance	(15 µg/plate)	-	14	22	16	17	4	0.9
Test substance	(50 µg/plate)	-	20	16	22	19	3	1.1
Test substance	(150 µg/plate)	-	12	16	16	15	2	0.8
Test substance	(500 µg/plate)	-	14	14	23	17	5	0.9
Test substance	(1500 µg/plate)	-	21	10	10	14	6	0.8
Test substance	(5000 µg/plate)	-	13	13	15	14	1	0.8
DMSO	(0.1 mL/plate)	+	19	21	20	20	1	-
Test substance	(5 µg/plate)	+	17	14	13	15	2	0.8
Test substance	(15 µg/plate)	+	8	24	15	16	8	0.8
Test substance	(50 µg/plate)	+	12	16	14	14	2	0.7
Test substance	(150 µg/plate)	+	17	14	14	15	2	0.8
Test substance	(500 µg/plate)	+	19	20	14	18	3	0.9
Test substance	(1500 µg/plate)	+	19	14	14	16	3	0.8
Test substance	(5000 µg/plate)	+	19	13	14	15	3	0.8
Sodium azide	(0.5 µg/plate)	-	342	293	195	277	75	15.4
2-Aminoanthracene	(2 µg/plate)	+	186	197	212	198	13	9.9
Viable cell count: 10^{-6} dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	223	177	216	205	25	-
Sterility checks (total colony counts):								
Buffer		-	0	0	0	0	0	-
S9 mix		+	0	0	0	0	0	-
Test substance	(5000 µg/plate)	-	0	0	0	0	0	-

TABLE 6: Results obtained with *S. typhimurium* TA1535: test 2

Tester strain: *S. typhimurium* TA1535
Test substance
Test No.: 2 (with pre-incubation)

Addition		S9 mix + present - absent	Revertant colony counts/plate					Ratio treated/ vehicle
			Individual plate counts			Mean	Standard Deviation	
DMSO	(0.1 mL/plate)	-	19	27	21	22	4	-
Test substance	(50 µg/plate)	-	19	31	28	26	6	1.2
Test substance	(150 µg/plate)	-	32	17	34	28	9	1.3
Test substance	(500 µg/plate)	-	17	22	29	23	6	1.0
Test substance	(1500 µg/plate)	-	19	21	17	19	2	0.9
Test substance	(5000 µg/plate)	-	19	16	17	17	2	0.8
DMSO	(0.1 mL/plate)	+	26	27	31	28	3	-
Test substance	(50 µg/plate)	+	24	21	19	21	3	0.8
Test substance	(150 µg/plate)	+	23	27	23	24	2	0.9
Test substance	(500 µg/plate)	+	27	30	23	27	4	1.0
Test substance	(1500 µg/plate)	+	28	28	30	29	1	1.0
Test substance	(5000 µg/plate)	+	22	21	22	22	1	0.8
Sodium azide	(0.5 µg/plate)	-	542	296	377	405	125	18.4
2-Aminoanthracene	(2 µg/plate)	+	165	199	196	187	19	6.7
Viable cell count: 10^{-6} dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	210	189	201	200	11	-
Sterility checks (total colony counts):								
Buffer		-	0	0	0	0	0	-
S9 mix		+	0	0	0	0	0	-
Test substance	(5000 µg/plate)	-	0	0	0	0	0	-

TABLE 7: Results obtained with *S. typhimurium* TA1537: test 1

Tester strain: *S. typhimurium* TA1537
Test substanc
Test No.: 1

Addition		S9 mix + present - absent	Revertant colony counts/plate					Ratio treated/ vehicle
			Individual plate counts			Mean	Standard Deviation	
DMSO	(0.1 mL/plate)	-	15	19	15	16	2	-
Test substance	(5 µg/plate)	-	19	16	13	16	3	1.0
Test substance	(15 µg/plate)	-	19	16	16	17	2	1.1
Test substance	(50 µg/plate)	-	19	21	20	20	1	1.3
Test substance	(150 µg/plate)	-	10	21	10	14	6	0.9
Test substance	(500 µg/plate)	-	20	12	16	16	4	1.0
Test substance	(1500 µg/plate)	-	20	14	17	17	3	1.1
Test substance	(5000 µg/plate)	-	10	10	14	11	2	0.7
DMSO	(0.1 mL/plate)	+	27	24	26	26	2	-
Test substance	(5 µg/plate)	+	20	21	28	23	4	0.9
Test substance	(15 µg/plate)	+	21	17	20	19	2	0.7
Test substance	(50 µg/plate)	+	19	22	21	21	2	0.8
Test substance	(150 µg/plate)	+	24	32	24	27	5	1.0
Test substance	(500 µg/plate)	+	30	28	23	27	4	1.0
Test substance	(1500 µg/plate)	+	20	24	24	23	2	0.9
Test substance	(5000 µg/plate)	+	28	23	28	26	3	1.0
9-Aminoacridine	(50 µg/plate)	-	2484	2084	1188	1919	664	119.9
Benzo[a]pyrene	(5 µg/plate)	+	342	383	347	357	22	13.7
Viable cell count: 10^{-6} dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	177	206	180	188	16	-
Sterility checks (total colony counts):								
Buffer		-	0	0	0	0	0	-
S9 mix		+	0	0	0	0	0	-
Test substance	(5000 µg/plate)	-	0	0	0	0	0	-

TABLE 8: Results obtained with *S. typhimurium* TA1537: test 2

Tester strain: *S. typhimurium* TA1537
Test substanc
Test No.: 2 (with pre-incubation)

Addition		S9 mix + present - absent	Revertant colony counts/plate					Ratio treated/ vehicle
			Individual plate counts			Mean	Standard Deviation	
DMSO	(0.1 mL/plate)	-	24	13	15	17	6	-
Test substance	(50 µg/plate)	-	13	20	16	16	4	0.9
Test substance	(150 µg/plate)	-	21	14	20	18	4	1.1
Test substance	(500 µg/plate)	-	19	13	13	15	3	0.9
Test substance	(1500 µg/plate)	-	17	13	14	15	2	0.9
Test substance	(5000 µg/plate)	-	20	13	17	17	4	1.0
DMSO	(0.1 mL/plate)	+	26	21	24	24	3	-
Test substance	(50 µg/plate)	+	22	23	30	25	4	1.0
Test substance	(150 µg/plate)	+	26	30	22	26	4	1.1
Test substance	(500 µg/plate)	+	26	22	29	26	4	1.1
Test substance	(1500 µg/plate)	+	23	27	24	25	2	1.0
Test substance	(5000 µg/plate)	+	26	21	16	21	5	0.9
9-Aminoacridine	(50 µg/plate)	-	649	595	601	615	30	36.2
Benzo[a]pyrene	(5 µg/plate)	+	263	245	186	231	40	9.6
Viable cell count: 10^{-6} dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	222	209	252	228	22	-
Sterility checks (total colony counts):								
Buffer		-	0	0	0	0	0	-
S9 mix		+	0	0	0	0	0	-
Test substance	(5000 µg/plate)	-	0	0	0	0	0	-

TABLE 9: Results obtained with *E. coli* WP2 *uvr*A (pKM101): test 1

Tester strain: *E. coli* WP2 *uvr*A (pKM101)
Test substance
Test No.: 1

Addition		S9 mix + present - absent	Revertant colony counts/plate					Ratio treated/ vehicle
			Individual plate counts			Mean	Standard Deviation	
DMSO	(0.1 mL/plate)	-	101	107	110	106	5	-
Test substance	(5 µg/plate)	-	107	86	90	94	11	0.9
Test substance	(15 µg/plate)	-	112	103	111	109	5	1.0
Test substance	(50 µg/plate)	-	90	110	102	101	10	1.0
Test substance	(150 µg/plate)	-	111	82	110	101	16	1.0
Test substance	(500 µg/plate)	-	84	108	124	105	20	1.0
Test substance	(1500 µg/plate)	-	106	125	97	109	14	1.0
Test substance	(5000 µg/plate)	-	111	102	116	110	7	1.0
DMSO	(0.1 mL/plate)	+	130	130	108	123	13	-
Test substance	(5 µg/plate)	+	125	140	110	125	15	1.0
Test substance	(15 µg/plate)	+	152	133	116	134	18	1.1
Test substance	(50 µg/plate)	+	124	139	139	134	9	1.1
Test substance	(150 µg/plate)	+	115	148	108	124	21	1.0
Test substance	(500 µg/plate)	+	121	109	132	121	12	1.0
Test substance	(1500 µg/plate)	+	116	118	114	116	2	0.9
Test substance	(5000 µg/plate)	+	117	111	118	115	4	0.9
AF-2†	(0.05 µg/plate)	-	656	557	568	594	54	5.6
2-Aminoanthracene	(10 µg/plate)	+	306	322	349	326	22	2.7
Viable cell count: 10^{-6} dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	177	219	205	200	21	-
Sterility checks (total colony counts):								
Buffer		-	0	0	0	0	0	-
S9 mix		+	0	0	0	0	0	-
Test substance	(5000 µg/plate)	-	0	0	0	0	0	-

† 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide

TABLE 10: Results obtained with *E. coli* WP2 *uvr*A (pKM101): test 2

Results obtained with *E. coli* WP2 *uvr*A (pKM101)
Test substance
Test No.: 2 (with pre-incubation)

Addition		S9 mix + present - absent	Revertant colony counts/plate					Ratio treated/ vehicle
			Individual plate counts			Mean	Standard Deviation	
DMSO	(0.1 mL/plate)	-	140	130	112	127	14	-
Test substance	(50 µg/plate)	-	140	102	130	124	20	1.0
Test substance	(150 µg/plate)	-	128	101	129	119	16	0.9
Test substance	(500 µg/plate)	-	132	115	111	119	11	0.9
Test substance	(1500 µg/plate)	-	123	99	129	117	16	0.9
Test substance	(5000 µg/plate)	-	128	121	97	115	16	0.9
DMSO	(0.1 mL/plate)	+	159	141	158	153	10	-
Test substance	(50 µg/plate)	+	166	135	176	159	21	1.0
Test substance	(150 µg/plate)	+	167	137	148	151	15	1.0
Test substance	(500 µg/plate)	+	125	166	158	150	22	1.0
Test substance	(1500 µg/plate)	+	145	164	176	162	16	1.1
Test substance	(5000 µg/plate)	+	161	175	146	161	15	1.1
AF-2†	(0.05 µg/plate)	-	443	470	457	457	14	3.6
2-Aminoanthracene	(10 µg/plate)	+	304	336	334	325	18	2.1
Viable cell count: 10^{-6} dilution of 10-hour bacterial culture, plated on nutrient agar (total colony counts)		-	230	213	238	227	13	-
Sterility checks (total colony counts):								
Buffer		-	0	0	0	0	0	-
S9 mix		+	0	0	0	0	0	-
Test substance	(5000 µg/plate)	-	0	0	0	0	0	-

† 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide

APPENDIX 1: Historical Control Data

Presented below are the historical control data from the period 1 May 1999 to 30 April 2004.

	TA100		TA1535		WP2 *uvr*A (pKM101)		TA98		TA1537	
S9 Mix	-	+	-	+	-	+	-	+	-	+
Maximum	217	235	35	38	280	273	57	79	45	54
Minimum	70	77	9	5	43	48	23	28	6	7
Mean	129	138	19	19	128	157	37	45	14	23
No. of values	621	643	616	638	569	591	623	645	607	628
Standard deviation	23	24	4	4	26	30	5	8	5	10
Upper 99% limit	219	237	35	38	283	276	58	80	46	55
Lower 99% limit	68	75	9	5	40	45	22	27	5	6

Positive Controls

	TA100		TA1535		WP2 *uvr*A (pKM101)		TA98		TA1537	
S9 Mix	-	+	-	+	-	+	-	+	-	+
	NaAz	B[a]P	NaAz	2-AA(2)	AF-2	2-AA(10)	2-NF	B[a]P	9-Aa	B[a]P
Maximum	1534	1373	1130	1145	3698	2305	4280	1070	3307	2115
Minimum	208	298	49	40	126	188	112	99	73	64
Mean	566	712	380	180	723	780	430	571	896	278
No. of values	1021	1045	1012	1035	966	992	1033	1052	424	1027

NaAz	Sodium azide 0.5 µg
AF-2	2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide 0.05 µg
2-NF	2-Nitrofluorene 1 µg
9-Aa	9-Aminoacridine 50 µg
B[a]P	Benzo[a]pyrene 5 µg
2-AA(2)	2-Aminoanthracene 2 µg
2-AA(10)	2-Aminoanthracene 10 µg

APPENDIX 2: Eye Research Centre GLP Compliance Statement 2003

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY

TEST TYPE

Analytical Chemistry
Ecosystems
Environmental Fate
Environmental Toxicity
Mutagenicity
Toxicology
Phys/Chem Tests

DATE OF INSPECTION
22nd April 2003

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander
25/7/03

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority